Time-gated biological imaging using colloidal quantum dots

M. Dahan[*§], T. Laurence*, F. Pinaud[*], D.S. Chemla*,
A.P. Alivisatos[†], M. Sauer[‡] and S. Weiss*

[*] *Lawrence Berkeley National Laboratory, Berkeley CA 94720,*

[†] *Department of Chemistry, University of California at Berkeley, CA 94720,*

[‡] *Physikalisch-Chemisches Institut, Universitat Heidelberg,*
Im Neuenheimer Feld 253, 69120 Heidelberg, Germany

[§] *Present address : Laboratoire Kastler Brossel, Ecole normale supérieure, 24 rue Lhomond,*
75231 Paris Cedex 05, France.

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e-mail: maxime.dahan@ens.fr (M.D.), alivis@uclink4.berkeley.edu (A.P.A), sauer@urz.uni-heidelberg.de (M.S.), sweiss@lbl.gov (S.W.).

The long (but not too long!) fluorescence lifetime of CdSe semiconductor nanocrystals quantum dots was exploited to enhance fluorescence biological imaging contrast and sensitivity by time-gated detection. Significant and selective reduction of the autofluorescence contribution to the overall image was achieved and enhancement of signal to background ratio by more than an order of magnitude was demonstrated.

Colloidal quantum dots (QDs) have recently been proposed as an alternative to conventional organic fluorophores because they offer distinct advantages (1, 2). Several different sizes of QDs, and hence colors of emission, can be excited simultaneously with a single excitation source. This enables straightforward signal multiplexing in biology and biotechnology applications where many channels of information must be observed. QDs are also more stable to repeated excitation, providing more photons and therefore higher sensitivity per measurement. An important unadressed area of difference between QDs and organic dyes concerns their fluorescence lifetime.

Common organic fluorophores emit from the first allowed singlet-singlet electronic transition in a few nanoseconds. Unfortunately, this prompt emission coincides with a high degree of short-lived autofluorescence background from many naturally occurring species in a biological specimen. Delayed (long lifetime) fluorescence labels for time-gated and fluorescence lifetime imaging (FLIM) (3) have long been sought for in order to enhance contrast in cellular imaging and analysis (4) and lanthanide chelates (5) with lifetimes in the submicrosecond to millisecond range have been successfully imaged by FLIM

(6). However, due to their 'too long' fluorescence lifetime, these probes have limited photon turnover rate and therefore limited sensitivity.

QDs are a type of fluorophore that emits light slow enough that most of the autofluorescence background is over by the time it emits, but fast enough to maintain high photon turnover rate. Therefore they may be ideal probes for spectrally multiplexed, time-gated cellular detection with enhanced selectivity and sensitivity.

We constructed a stage-scanning, time correlated single photon counting (TCSPC) confocal microscope (Fig. 1) based on a closed-loop piezo-scanner (P-517.3CL, Physik Instrumente) that allows nanometer accuracy stepping of the specimen with respect to the stationary, on-axis and diffraction-limited confocal spot (7). A pulsed excitation at 503 nm a 5 MHz repetition rate was provided by a Ti:Saph laser (Coherent, Mira 900) operating at 1006 nm (pulse width 150 fs, FWHM) followed by a pulse picker and a doubling crystal or by a blue (405 nm) picosecond GaN diode laser (LDH 400, PicoQuant, Germany). Fluorescence was collected with a high NA objective (Zeiss Apochromat 1.3, 100x), passed through an optical band pass filter and focused onto a single-photon avalanche photodiode (APD, SPCM AQ141, EG&G, Perkin Elmer). At each piezo-stage step, the scanning controller board output a TTL pulse triggering the acquisition of the detected fluorescence photons by a PC plug-in TCSPC card (TimeHarp, PicoQuant, Germany) working in the continuous mode. The overall temporal response function of the system was measured to be 300 ps. This data acquisition scheme produces histograms of photon arrival-times for each pixel in the image.

These histograms can then be manipulated and analyzed in various ways as, for example, in FLIM and time-gated imaging applications.

The ensemble fluorescence lifetime of yellow QDs (18 Å radius, 575 nm peak emission, quantum yield ~ 40 %) in 1-butanol is presented in Fig.2. The fluorescence decay shows a strong non-exponential behavior that can be satisfactorily described by a sum of three exponents with time constants 3.4 (0.3), 16.1 (0.4) and 35.6 (0.8) ns (and amplitudes corresponding respectively to 1, 50 and 48 % of the emitted photons). The origin of these non exponential decays is currently under investigation. For cell staining experiments, we used silanized nanocrystals (1) that displayed a slightly faster decay and a small decrease in quantum yield compared to core-shell QDs in butanol. They maintained, however, the non-exponential behavior with components around 2, 8 and 24 ns (11, 49 and 40% of the fluorescence), still significantly longer than the autofluorescence decay (2-3 ns) and conventional dyes fluorescence lifetime (1-5 ns).

3T3 mouse fibroblasts were grown on fibronectin treated coverslips. A small amount of silanized nanocrystals (at concentration ~ 10-100 nM) was added to the growth medium and incubated overnight. After the incubation was completed, the cells were rinsed, clean medium (not containing QDs) was added and they were allowed to grow for another 3 hours before fixation. We did not observe any signs of toxic response to the presence of QDs in the medium even after uptake by the cells. Cells were then fixed in a mixture of formaldehyde (4%) and glutaraldehyde (0.25 %) at room temperature and mounted on a microscope slide.

A time-resolved confocal image of a fixed 3T3 cell stained with QDs is shown in Fig. 3. The unprocessed image, with all detected photons, is displayed in Fig. 3a while Fig. 3b shows the same image constructed only from photons arriving in the time window 35 to 65 ns after the laser pulse. Compared to the raw data, we observe mostly bright, localized spots with enhanced signal to background ratio. These spots are aggregation of QDs inside the cytosol, most likely uptaken by the cell via endocytosis and possibly stored in the lysozomes. In contrast to foreign organic compounds which are degraded, the inorganic QDs are stable in this environment and can be stored in the cell and watched for long periods of time.

The enhancement in signal to background is illustrated in an image line cross-section (insets). Although Fig. 3b show a significant reduction in the total number of photons in each pixel, the cross-section shows a background-free detection of QDs. To estimate the change in signal to background, a part of the image was selected and considered as background photons while the counts in a couple of bright spots were averaged and used as a measure of the signal. For the parameters used here (gating between 35 and 65 ns), the gain in signal to background is about 15.

This enhancement in image contrast demonstrates the potential of QDs for high sensitivity biological imaging and brings us closer to the detection limit of a single QD in live cells. Together with spectral multiplexing, nanometer accuracy co-localization (8) and reduced photobleaching, QD labels might offer unique and novel tools for cellular analysis.

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Figure Captions:

Fig. 1: Experimental setup. The microscope is a homemade stage-scanning confocal microscope using a nanometer-resolution closed-loop piezo-stage scanner (PS). A single laser line is brought via a fiber and a beam expander to the back focal plane of the objective (Ob) after reflection on a dichroic mirror (DC). Fluorescence is collected by the same objective and focused onto a pinhole (PH) via a tube lens (TL). The pinhole is imaged onto the APD with another imaging lens (IL). The start and stop pulses are provided by the APD and the laser electronics respectively (reverse mode).

Fig. 2: Normalized ensemble fluorescence decay of CdSe nanocrystals (18 Å radius, 575 nm peak emission). The solid dark line is a triple exponential fit to the curve with components at 3.4, 16.1 and 35.6 ns. The measured fluorescence decay of Rhodamine molecules in water is also displayed (dashed line) and is well described by a single exponential decay with a time constant 4.3 ns.

Fig. 3: Time-resolved confocal image of a fixed 3T3 cell: (a) Image obtained from all the detected photons, (b) Gated image constructed only from photons arrived between 35 and 65 ns after the laser pulse. The image was taken with a laser intensity of 0.1 kW/cm^2 and 25 ms integration time per pixel. The scale bars are 5 μm. The insets show a cross-section along the same horizontal line (indicated by the black arrows) for (a) and (b).

Figure 1 – Dahan et al.

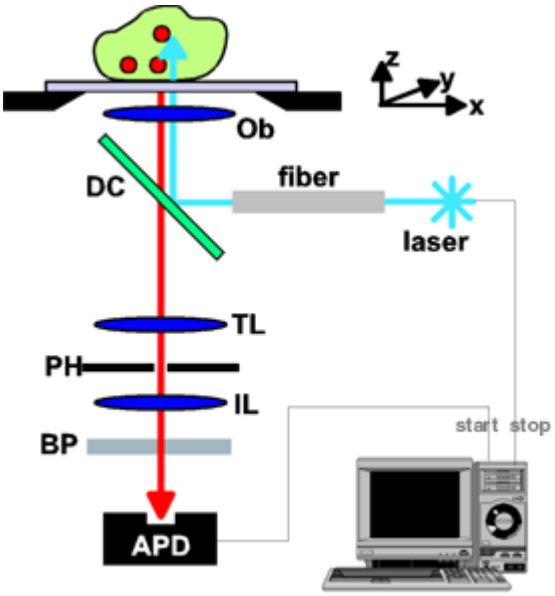

Figure 2 – Dahan et al.

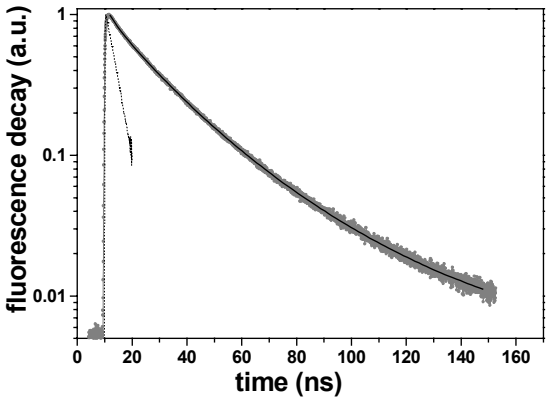

Figure 3 – Dahan et al.